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(54) **MODIFIED HYDROXPOLYMER CONJUGATES WITH KILLING EFFECT ON TUMOR CELLS**

MODIFIZIERTE HYDROXPOLYMER-KONJUGATE MIT ABTÖTENDER WIRKUNG AUF
TUMORZELLEN

CONJUGUÉS D'HYDROXPOLYMÈRE MODIFIÉ À ACTIVITÉ DESTRUCTRICE DE CELLULES
TUMORALES

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Description

Technical Field of Invention

[0001] The present invention is related to a modified hydroxypolymer conjugate, particularly a guanidine-dextran conjugate having a tumor killing effect, and a method for preparation of said conjugates as well as its use.

Background of Invention

[0002] Guanidine compounds have many interesting and important biomedical effects and consequently many uses. They are for example used as free radical scavengers, inhibitors of nitric oxide synthase (NOS) preventing nitric oxide formation. They are known to have certain anti-proliferative effects on cells. The guanidines are also used as "anti-aging" drugs, wherein the effect is achieved by protection from low density lipoprotein oxidation, by prevention of atherosclerotic lesions formation, by retarding the tissue damaging effects of diabetes, e.g. cardiovascular damage, through inhibition of the formation of advanced glycosylation end products (AGE's) as described in the following scientific publications (Yildiz et al, Br J Pharmacol, 1998 Jul; 124 (5): 905-910; Scaccini et al, J Lipid Res, 1994, Vol 35, 1085-1092; Higashi et al, J Biochem, 2004, Vol 136, No 4, 533-539; Szende et al, Cancer Cell Int, 2001, 1:3; Sell et al, J Gerontol Soc Am, 2001, 56: B405-B411; Wang et al, World J Gastroenterol. 2005, Jul 7:11, (25) 3830-3833; Zhang et al, World J Gastroenterol. 2001, June 7, 3) :331-334;JP Beisswenger, Dartmouth Med, http://dartmed.dartmouth.edu/summer00/html/bench_to_bedside.shtml. Aminoguanidine is currently supplied as a non-prescription drug in the United States and its role in the treatment of various diseases is investigated in clinical studies. The inhibitory effect of agmatine on proliferation of tumor cells by modulation of polyamine metabolism is described in Wang et al., Acata Pharmacologica Sinica 2005, May; 26(5): 616-622.

[0003] Since more than five decades dextran is an established pharmaceutical used for preventing hypovolemic shock, preventing embolism and improving microcirculation. Dextran and its non-toxic properties and high tolerability is very well documented (AS Segal, 1964 In: Modern medical monographs, ed. Wright, IS, Green & Stratton, NY, London, pp 5-17. Therefore, it is often used as an example of pharmaceutically applicable hydroxypolymers.

Summary of Invention

[0004] Guanidine compounds and dextrans have almost no toxic effect on tumor cells as shown in Figure 1. However, it was surprisingly found that when the guanidine compounds were covalently conjugated to an activated hydroxypolymer, the conjugates demonstrated a high antitumor efficacy, which was similar or at some

dosages even higher than that of conventional antitumor drugs, like Adriamycin®.

[0005] Accordingly, the present invention is related to a modified hydroxypolymer conjugate having a tumor cell killing effect. The modified hydroxypolymer conjugate is a hydroxypolymer substituted with guanidine compounds, which are covalently coupled to activated hydroxyl groups of the hydroxypolymer moiety.

[0006] The guanidine compounds in the present invention are agmatine or aminoguanidine and the hydroxypolymer moiety is a dextran, which preferably has a molecular weight in the range 10^3 - 10^6 Daltons and which is activated before being substituted with said guanidine compounds.

[0007] The modified hydroxypolymer conjugate may further comprise covalently coupled functional groups, including radio-nuclides, therapeutic compounds, anticancer drugs, targeting agents, etc.

[0008] The modified hydroxypolymer conjugate is preferably a guanidine-dextran conjugate having an average molecular weight of approximately $70 \text{ kD} \pm 25 \text{ kD}$, wherein 15-60%, preferably 20-50% of the glucose moieties of the dextran are substituted with guanidine compounds having at least one free amino side group, Said guanidine compounds, agmatine or aminoguanidine, are covalently coupled to activated hydroxyl groups of the said dextran. The hydroxypolymer conjugates may further comprise covalently coupled functional groups, including radio-nuclides, therapeutic compounds, anticancer drugs, targeting agents, etc. Useful targeting agents are for example biphosphonates, e.g. alendronate.

[0009] The present invention is also related to the use of said hydroxypolymer conjugates as medicines, particularly for manufacturing medicines for treating tumors. The medicines are tumor killing compositions, which comprise the modified hydroxypolymer conjugate and at least pharmaceutically acceptable adjuvant. The medicines or tumor killing compositions are locally, intravesically, regionally, peritoneally, intratumorally, systemically or intravenously administrable. The medicines may further comprise covalently coupled functional groups, including radio-nuclides, therapeutic compounds, anticancer drugs, targeting agents, etc.

[0010] The present invention also provides a method for producing the modified hydroxypolymer conjugate. In the present method a guanidine compound, having at least one free amino group, which is agmatine or aminoguanidine, is mixed with an eluent obtained when purifying an activated hydroxypolymer by gel filtration. The hydroxypolymer is activated by an oxidative reaction, wherein a periodate salt is added to an aqueous solution comprising said hydroxypolymer with subsequent addition of concentrated sulphuric acid and by ending the reaction by adding ethylene glycol. The hydroxypolymer, which has been activated as described above, is subsequently purified by gel filtration.

Description of Drawings

[0011]

Figure 1 demonstrates the toxic effect of dextran and aminoguanidine as compared to anthracycline = Adriamycin® using living urothelial cancer cells (RT4, ATCC, Manassas, USA). Percent survival is plotted on the y axes and incubation concentration is plotted on the x axes.

Figure 2 shows the toxic effect of Guanidine-Dextran conjugates amgdx = aminoguanidine-dextran, agmdx = agmatine-dextran in comparison to anthracycline = Adriamycin® on urothelial cancer cells (RT4, ATCC, Manassas, USA). Percent survival is plotted on the y axes and incubation concentration is plotted on the x axes. The survival of urothelial cancer cells is $\leq 1 = 0$. The lower value means 100% cell death.

Figure 3 shows the toxic effect of Guanidine-Dextran conjugates amgdx = aminoguanidine-dextran, agmdx = agmatine-dextran as compared to anthracycline. = Adriamycin® on urothelial cancer cells (5637, ATCC, Manassas, USA). Percent survival is plotted on the y axes and incubation concentration is plotted on the x axes. The survival of urothelial cancer cells is $\leq 1 = 0$.

Figure 4 shows the toxic effect of Guanidine-Dextran conjugates amgdx = aminoguanidine-dextran, agmdx = agmatine-dextran as compared to anthracycline. = Adriamycin® on prostate cancer cells (PC3, ATCC, Manassas, USA). The survival of prostate cancer cells is $\leq 1 = 0$.

Figure 5 shows the toxic effect of Guanidine-Dextran conjugates amgdx = aminoguanidine-dextran, agmdx = agmatine-dextran as compared to anthracycline. = Adriamycin® on breast cancer cells (MDA231, ATCC, Manassas, USA). The survival of breast cancer cells is $\leq 1 = 0$.

Figure 6 shows the toxic effect of Guanidine-Dextran conjugates amgdx = aminoguanidine-dextran, agmdx = agmatine-dextran as compared to anthracycline. = Adriamycin® on renal cell cancer (A498, ATCC, Manassas, USA). The survival of renal cancer cells is $\leq 1 = 0$ survival.

Detailed Description of Invention

[0012] The inventors have discovered that certain guanidine compounds, having very low intrinsic toxicity, when covalently conjugated to a non-toxic hydroxyl polymer, viz dextran, have a high tumor cell killing efficacy. The tumor cell killing efficacy is equal to established an-

thracycline anticancer pharmaceuticals such as Farnorubicine® and Adriamycine® or at some concentrations higher than established anticancer drugs. It appears probable that due to the harmless nature of the constituents of said polymer-guanidine conjugates, the handling of such conjugates i.e. for medical staff and the treatment connected side-effects for the patients can be expected to be favourable in comparison to conventional chemotherapeutic anti-cancer pharmaceuticals.

[0013] Targeting or tumor cell selectivity is believed to be achieved through the increased metabolic needs of the fast dividing tumor cells, which require amino-5-guanidinopentanoic acid and structurally related guanidine compounds, and other constituents for polyamine formation (D Scott Lind, Am Soc Nutr Sci, 2004, J Nutr, 134: 2837-2841; E Wayne, Turk J Med Sci, 2003, 33, 195-205). Cellular uptake of said guanidine compounds is mediated principally via the Na⁺-independent basic amino acid transport system y⁺ (Cendan et al, Ann Surg Oncol, 1995, 2:257-265).

[0014] The guanidine-polymer-conjugate of the present inventions is a compound, which as a backbone compound has a hydroxypolymer which is dextran. The molecular weight of the dextran is between 10³-10⁶ Daltons. Typically, the dextran is substituted with between 15 to 60%, preferably between 20 to 50% guanidine side groups, i.e. 20-50% of the glucose moieties in the dextran backbone are substituted with guanidine groups.

[0015] In one analyzed batch, it was demonstrated that 100 mg aminoguanidine-dextran-conjugate, where the dextran polymer has a mean molecular weight of 70 kD, about 30 mg constitutes guanidine side groups. In other words, 273 μ mol aminoguanidine is coupled to 1.4 μ mol of dextran70, which means that about 50 % of the glucose moieties are substituted with a guanidine group. In another batch, it was found that 100 mg aminoguanidine-dextran-conjugate, where the dextran polymer has a mean molecular weight of 70 kD, about 10 mg constitutes guanidine side groups. In other words, 90 μ mol aminoguanidine is coupled to 1.3 μ mol of dextran70, which means that about 18% of the glucose moieties are substituted with a guanidine group.

[0016] The medicine is preferably administered locally, e.g. by intra-vesical administration, regiolocally, e.g. by administration to the peritoneal cavity, intratumorally, i.e. by injection directly into a solid tumor, or systemically, i.e. by intra-venous administration. All these routes of administration are possible and dependent on the specific therapeutic application.

[0017] The dextran polymer is substituted using side groups of guanidine. The guanidine groups are covalently coupled to said dextran polymer via free amino side groups of the guanidine compounds. The substitution is preceded by activating said dextran polymer through oxidation, which enables the reaction with said free amino side groups forming bonds of guanidine compounds with subsequent reduction obtain stable amine bonds (Mat-sunaga et al, Nucl Med Biol. 2005, 32, 279-285).

[0018] In the exemplified case, the aminoguanidine forms a hydrazone bond with the dextran polymer. The hydrazone bond can be further reduced, yielding a guanidino hydrazine bond. The guanidine compounds are agmatine and aminoguanidine.

[0019] Other functional groups may be coupled additionally to said guanidine hydroxypolymer conjugate. These functional groups include therapeutic drugs, radionuclides, such as. Tc-99m, I-131, Y-90, Re-188, Sm-153, hormones, hormone antagonists, such as Tamoxifen, peptides, such as somatostatin, toxins, monoclonal antibodies or fragments thereof, free radical scavengers, such as amifostine or proteins and further targeting agents. Coupling of such functional groups is achieved directly or via bifunctional chelates that have been coupled to the hydroxypolymer prior to the inclusion of functional groups.

Example 1

Dextran activation

[0020] Pharmaceutical grade dextran PM70 (150 mg) was dissolved in 6 ml of water. Sodium periodate 120 mg, was added, followed by 25 μ l of concentrated sulphuric acid. The combined solution was stirred for 30 minutes. Ethylene glycol, 75 μ l, was added in order to stop the oxidation and destroy excess periodate, by reacting for a further 15 minutes. The activated dextran was purified from low-molecular components by gel filtration in one ml portions on PD-10 columns with 0.1 M acetate, pH 6.5, as an eluent. In each separation, 2 ml of activated dextran solution was obtained.

Conjugation

[0021] The eluent, 1 ml, obtained by the procedure above, was mixed with

- A) agmatine sulphate, 110 mg, and sodium cyanoborohydride, 5 mg,
- B) aminoguanidine hydrochloride, 65 mg.

[0022] The solutions were stirred over night at room temperature and separated from low-molecular components on PD 10 columns with PBS as eluent. From each reaction, two ml of purified conjugate solution was obtained.

[0023] Fluorometric Cytotoxicity Assay (Figures 1-6)

[0024] The fluorometric cytotoxicity assay was performed as described by Larsson and Nygren (Larson et al, Anticancer Res, 1989, 9:1111-1119).

[0025] Briefly, approximately 10,000 cells/well were seeded (96-well microtiter plates, Falcon, Becton Dickinson, Meylan, France). Anthracycline and guanidine-dextran-conjugate with aminoguanidine or agmatine groups were added at equimolar concentrations (0.1, 0.5, 1, 2, and 4 μ M). The control wells were given the same

amount of PBS. After 72 h incubation the microtiter plates were centrifuged (200 x g for 3 minutes) and the medium was removed by flicking the plates. The cells were washed in PBS. Fluorescein diacetate (FDA, Sigma, Stockholm, Sweden) was dissolved in DMSO and kept frozen at -20°C as a stock solution (10 mg/ml). The FDA stock solution was diluted in PBS at a concentration of 10 μ g/ml and 200 μ l was added to each well. The plates were then incubated for 30 minutes at 37°C. A 96-well scanning fluorometer was used to count the emitted fluorescence from living cells. The data were transferred to a computer and the results were calculated. The results are shown in Figures 1-5, y axes showing % cell survival and x axes showing the molarity of the test substances.

[0026] The following tumor cell-lines were used (from ATCC, Manassas, US): RT4, 5637 (Urothelial cancer), MDA231 (Breast cancer), PC3 (Prostate cancer), A498 (Renal cell cancer).

Claims

1. A modified hydroxypolymer conjugate having a tumor cell killing effect, **characterized in that** the modified hydroxypolymer conjugate is a hydroxypolymer dextran substituted with guanidine compounds agmatine or aminoguanidine, which have at least one free amino group and which are covalently coupled to activated hydroxyl groups of said hydroxypolymer moiety.
2. The modified hydroxypolymer conjugate according to claim 1, **characterized in that** said hydroxypolymer dextran has a molecular weight of 10^3 - 10^6 Daltons.
3. The modified hydroxypolymer conjugate according to claim 1, **characterized in that** the modified hydroxypolymer conjugate further comprises at least one covalently coupled functional group.
4. The modified hydroxypolymer according to claim 3, **characterized in that** said functional group is a radio-nuclide.
5. The modified hydroxypolymer according to claim 3, **characterized in that** said functional group is a therapeutic compound.
6. The modified hydroxypolymer according to claim 3, **characterized in that** said functional group is an anticancer drug.
7. The modified hydroxypolymer according to claim 3, **characterized in that** said functional group is a further targeting agent.
8. The modified hydroxypolymer conjugate according

to claim 1, **characterized in that** the modified hydroxypolymer conjugate is a guanidine-dextran conjugate having an average molecular weight of approximately 70 kD $\pm$ 25 kD and wherein 15-60%, preferably 20-50 % of the glucose moieties of the dextran are substituted with guanidine compounds agmatine or aminoguanidine having at least one free amino side group, said guanidine compounds being covalently coupled to activated hydroxyl groups of the said dextran.

9. The modified hydroxypolymer conjugate according to any of claims 1-8, **characterized in that** said modified hydroxypolymer conjugate is locally, intravesically, regiolocally, peritoneally, intratumorally, systemically or intravenously administrable.

10. A composition having a tumor cell killing effect, **characterized in that** said composition comprises a hydroxypolymer dextran substituted with guanidine compounds agmatine or aminoguanidine, which have at least one free amino group and which are covalently coupled to activated hydroxyl groups of said dextran moiety and at least one pharmaceutically acceptable adjuvant.

11. A method for producing the modified hydroxypolymer conjugate according to claim 1, **characterized in that** a guanidine compound agmatine or aminoguanidine having at least one free amino group is mixed with an eluent comprising an activated dextran purified by gel filtration.

12. The method according to claim 11, **characterized in that** the activated dextran is obtained by an oxidative reaction, wherein a periodate salt is added to an aqueous solution comprising dextran with subsequent addition of concentrated sulphuric acid and wherein the oxidative reaction is stopped by adding ethylene glycol, followed by purification with gel filtration.

Patentansprüche

1. Modifiziertes Hydroxypolymer-Konjugat mit einem Tumorzellenabtötungseffekt, **dadurch gekennzeichnet, dass** das modifizierte Hydroxypolymer-Konjugat ein Hydroxypolymer-Dextran, substituiert mit den Guanidinverbindungen Agmatin oder Aminoguanidin, ist, die mindestens eine freie Aminogruppe aufweisen und die mit aktivierten Hydroxylgruppen des Hydroxypolymeranteils kovalent gekoppelt sind.

2. Modifiziertes Hydroxypolymer-Konjugat nach Anspruch 1, **dadurch gekennzeichnet, dass** das Hydroxypolymer-Dextran ein Molekulargewicht von

10³-10⁶ Dalton aufweist.

3. Modifiziertes Hydroxypolymer-Konjugat nach Anspruch 1, **dadurch gekennzeichnet, dass** das modifizierte Hydroxypolymer-Konjugat ferner mindestens eine kovalent gekoppelte funktionale Gruppe umfasst.

4. Modifiziertes Hydroxypolymer nach Anspruch 3, **dadurch gekennzeichnet, dass** die funktionale Gruppe ein Radionuklid ist.

5. Modifiziertes Hydroxypolymer nach Anspruch 3, **dadurch gekennzeichnet, dass** die funktionale Gruppe eine therapeutische Verbindung ist.

6. Modifiziertes Hydroxypolymer nach Anspruch 3, **dadurch gekennzeichnet, dass** die funktionale Gruppe ein Antikrebs-Medikament ist.

7. Modifiziertes Hydroxypolymer nach Anspruch 3, **dadurch gekennzeichnet, dass** die funktionale Gruppe ein weiteres Zielmittel ist.

8. Modifiziertes Hydroxypolymer-Konjugat nach Anspruch 1, **dadurch gekennzeichnet, dass** das modifizierte Hydroxypolymer-Konjugat ein Guanidin-Dextran-Konjugat mit einem mittleren Molekulargewicht von ungefähr 70 kD $\pm$ 25 kD ist, und wobei 15-60 %, vorzugsweise 20-50 % der Glucoseanteile des Dextrans mit den Guanidinverbindungen Agmatin oder Aminoguanidin mit mindestens einer freien Aminoseitengruppe substituiert sind, wobei die Guanidinverbindungen mit aktivierten Hydroxylgruppen des Dextrans kovalent gekoppelt sind.

9. Modifiziertes Hydroxypolymer-Konjugat nach einem der Ansprüche 1-8, **dadurch gekennzeichnet, dass** das modifizierte Hydroxypolymer-Konjugat lokal, intravesikal, regiolokal, peritoneal, intratumoral, systemisch oder intravenös verabreicht werden kann.

10. Zusammensetzung mit einem Tumorzellenabtötungseffekt, **dadurch gekennzeichnet, dass** die Zusammensetzung ein Hydroxypolymer-Dextran umfasst, das mit den Guanidinverbindungen Agmatin oder Aminoguanidin substituiert ist, die mindestens eine freie Aminogruppe aufweisen und die mit aktivierten Hydroxylgruppen des Dextrananteils und mindestens einem pharmazeutisch verträglichen Wirkverstärker kovalent gekoppelt sind.

11. Verfahren zur Herstellung des modifizierten Hydroxypolymer-Konjugats nach Anspruch 1, **dadurch gekennzeichnet, dass** eine Guanidinverbindung Agmatin oder Aminoguanidin mit mindestens einer freien Aminogruppe mit einem Elutionsmittel mit ei-

nem aktivierten Dextran, das durch Gelfiltration gereinigt wird, vermischt wird.

12. Verfahren nach Anspruch 11, **dadurch gekennzeichnet, dass** das aktivierte Dextran durch eine Oxidationsreaktion erhalten wird, wobei ein Perjodatsalz zu einer wässrigen Lösung zugegeben wird, die Dextran enthält, mit anschließender Zugabe von konzentrierter Schwefelsäure, und wobei die Oxidationsreaktion durch Zugabe von Ethylenglycol gestoppt wird, gefolgt von Reinigung mit Gelfiltration.

Revendications

1. Conjugué d'hydroxypolymère modifié ayant une action destructrice des cellules tumorales, **caractérisé par le fait que** le conjugué d'hydroxypolymère modifié est un hydroxypolymère dextrane substitué par des composés guanidines agmatine ou aminoguanidine, qui ont au moins un groupe amino libre et qui sont couplés de façon covalente à des groupes hydroxyle activés de ladite fraction hydroxypolymère.
2. Conjugué d'hydroxypolymère modifié selon la revendication 1, **caractérisé par le fait que** ledit hydroxypolymère dextrane a une masse moléculaire de 10^3 - 10^6 Daltons.
3. Conjugué d'hydroxypolymère modifié selon la revendication 1, **caractérisé par le fait que** le conjugué d'hydroxypolymère modifié comprend en outre au moins un groupe fonctionnel couplé de façon covalente.
4. Hydroxypolymère modifié selon la revendication 3, **caractérisé par le fait que** ledit groupe fonctionnel est un radionucléide.
5. Hydroxypolymère modifié selon la revendication 3, **caractérisé par le fait que** ledit groupe fonctionnel est un composé thérapeutique.
6. Hydroxypolymère modifié selon la revendication 3, **caractérisé par le fait que** ledit groupe fonctionnel est un médicament anti-cancer.
7. Hydroxypolymère modifié selon la revendication 3, **caractérisé par le fait que** ledit groupe fonctionnel est un autre agent de ciblage.
8. Conjugué d'hydroxypolymère modifié selon la revendication 1, **caractérisé par le fait que** le conjugué d'hydroxypolymère modifié est un conjugué guanidine-dextrane ayant une masse moléculaire moyenne d'approximativement $70 \text{ kD} \pm 25 \text{ kD}$ et dans lequel 15-60 %, de préférence 20-50 %, des

fractions glucose du dextrane sont substituées par des composés guanidines agmatine ou aminoguanidine ayant au moins un groupe latéral amino libre, lesdits composés guanidines étant couplés de façon covalente à des groupes hydroxyle activés dudit dextrane.

9. Conjugué d'hydroxypolymère modifié selon l'une quelconque des revendications 1 à 8, **caractérisé par le fait que** ledit conjugué d'hydroxypolymère modifié est administrable par voie locale, intravésicale, régiolocale, péritonéale, intratumorale, systémique ou intraveineuse.
10. Composition ayant une action destructrice des cellules tumorales, **caractérisée par le fait que** ladite composition comprend un hydroxypolymère dextrane substitué par des composés guanidines agmatine ou aminoguanidine, qui ont au moins un groupe amino libre et qui sont couplés de façon covalente à des groupes hydroxyle activés de ladite fraction dextrane et au moins un adjuvant pharmaceutiquement acceptable.
11. Procédé de fabrication du conjugué d'hydroxypolymère modifié selon la revendication 1, **caractérisé par le fait qu'un** composé guanidine agmatine ou aminoguanidine ayant au moins un groupe amino libre est mélangé avec un éluant comprenant un dextrane activé purifié par filtration sur gel.
12. Procédé selon la revendication 11, **caractérisé par le fait que** le dextrane activé est obtenu par une réaction d'oxydation, un sel periodate étant ajouté à une solution aqueuse comprenant du dextrane avec addition ultérieure d'acide sulfurique concentré, et la réaction d'oxydation étant stoppée par addition d'éthylène glycol, en faisant suivre par une purification par filtration sur gel.

Fig 1

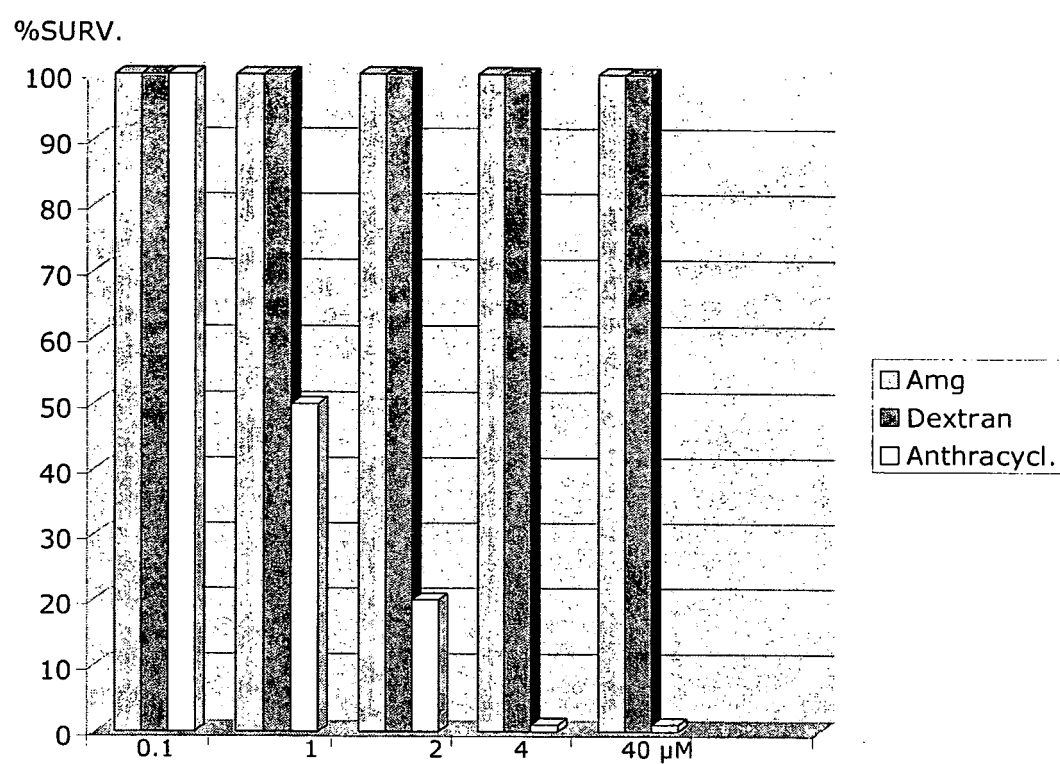


Fig 2

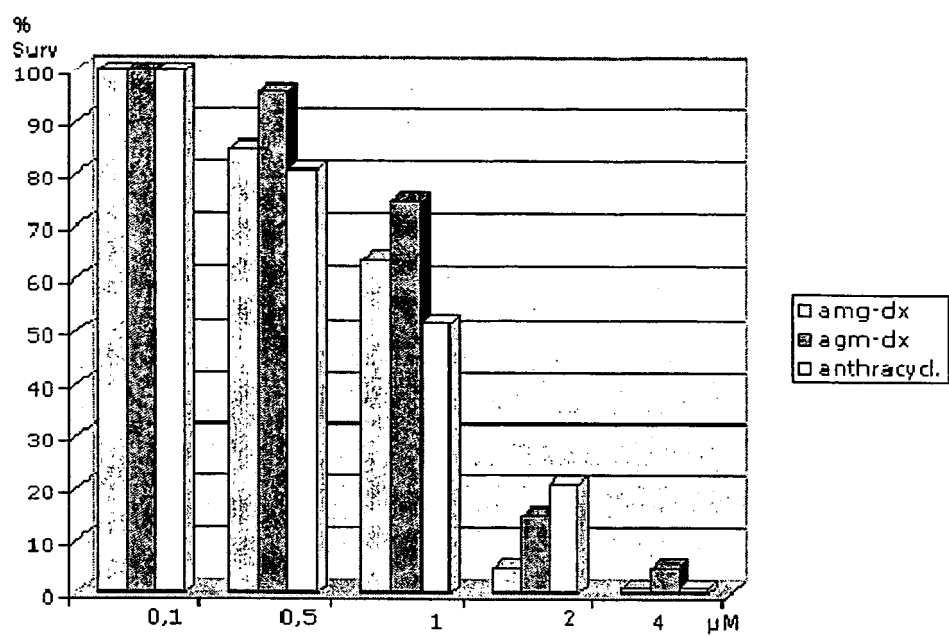


Fig 3

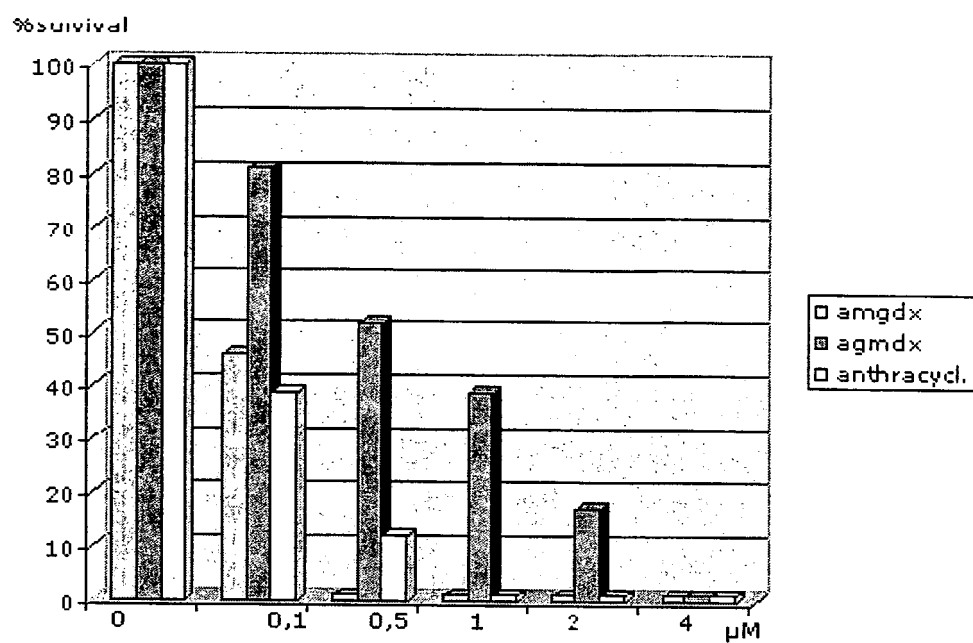


Fig 4

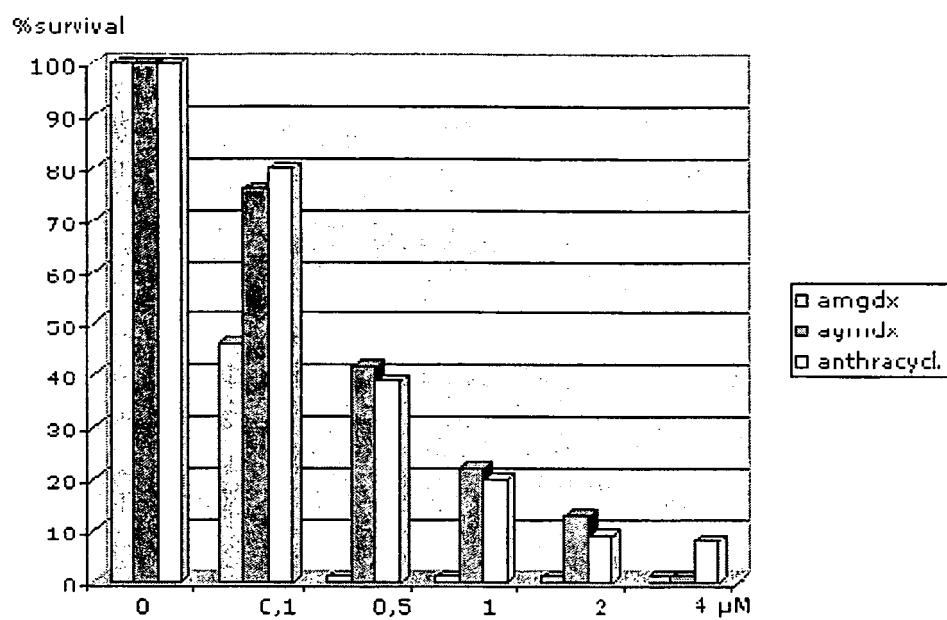
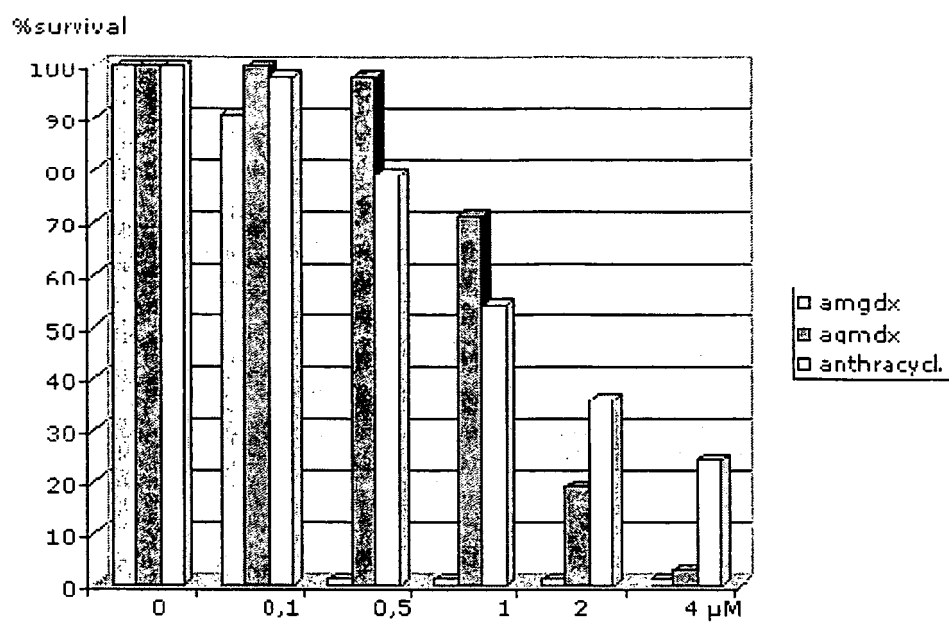
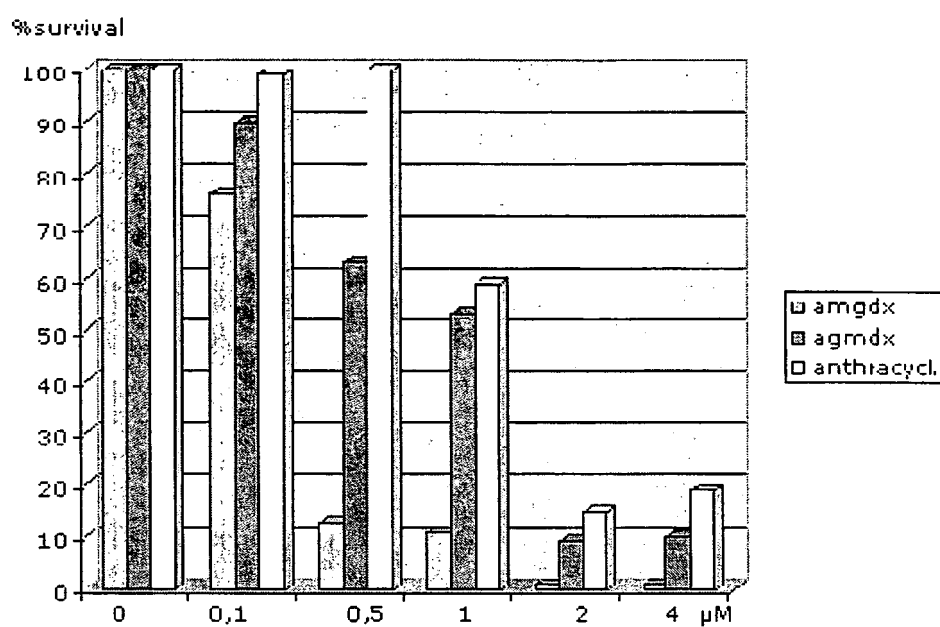


Fig 5



- Fig 6



REFERENCES CITED IN THE DESCRIPTION

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